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Linking Innate and Adaptive Immunity to *Streptococcus pneumoniae*

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Cover: Transmission electron microscopy picture of a human dendritic cell infected with *Streptococcus pneumoniae*.

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Science (from the Latin *scientia*, meaning "knowledge") is an enterprise that builds and organizes knowledge in the form of testable explanations and predictions about the natural world.

Science is proud to make predictions with great probability, bearing in mind that the most likely event is not always what actually happens.

ABSTRACT

Streptococcus pneumoniae (the pneumococcus) most commonly colonizes the human nasopharyngeal mucosa without causing any symptoms. However, this organism has the potential to spread to normally sterile sites and cause pneumonia, meningitis or sepsis; diseases which are characterized by excessive inflammation. Despite the large burden of pneumococcal disease, relatively little is known about the mechanisms behind development of natural immunity to the pneumococcus. The purpose of this thesis was to study the role of human dendritic cells (DCs) in linking innate and adaptive immune responses to pneumococci. The immunological events in which DC-mediated T helper (Th) cell responses are generated were also investigated, as well as possible ways to modulate these responses.

As a first part of this work, a novel role of the pneumococcal toxin pneumolysin in the evasion of DC-mediated immunosurveillance was described. Pneumolysin inhibited DC maturation, production of inflammatory cytokines and inflammasome activation, and induced caspase-dependent apoptosis of infected cells. Interestingly, murine DCs differed in their response to pneumolysin, emphasizing the need to study human responses to this human-specific pathogen.

In the second part of this work, we demonstrated that pneumococcus-infected monocytes and DCs efficiently promote the production of inflammatory Th1 and Th17 cytokines from autologous co-cultured memory cells. Live pneumococci and pneumococcal peptidoglycan triggered activation of DCs, which in turn induced the generation of Th cytokines via cell-to-cell contact and soluble components. Our work further revealed that the inflammatory response could be modulated with exogenous substances, such as recombinant cytokines, and cytokine- and receptor-blocking antibodies. Moreover, exposure of DCs to vitamin D skewed the response from an inflammatory Th1/Th17 phenotype towards a regulatory T cell phenotype.

In the last part of this work, we focused on patients with primary immunodeficiencies (PIDs), suffering from frequent respiratory tract infections. The mechanisms behind the infectious susceptibility among these patients remain elusive and we hypothesized that it may be due to defects in the production of antimicrobial peptides (AMPs) in the nasal mucosa. We found that two patient groups, namely common variable immunodeficiency (CVID) and Hyper-IgE syndrome (HIES), had a dysregulated AMP response to bacteria in the upper respiratory tract. In addition, cells from these patients exhibited an impaired Th17 cytokine response.

In order to improve management of patients with pneumococcal infections there is a need to elucidate the role of DC-mediated cytokine responses in the delicate balance between protective immunity and immunopathology. An increased understanding of these processes is also essential for the development of pneumococcal vaccines, designed to elicit cell-mediated immunity. The work presented in this thesis contributes to our understanding of the dynamic interplay between pneumococci and host cells, and provides the opportunity to explore the potential role of vitamin D in limiting the inflammatory response in pneumococcal disease.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Uppskattningsvis dör årligen en miljon barn under fem års ålder av sjukdomar orsakade av bakterien *Streptococcus pneumoniae*, eller **pneumokocker** som de kallas i dagligt tal. Sedan några år tillbaka vaccineras barn mot pneumokocker inom barnvaccinationsprogrammet. De vaccin som finns idag skyddar dock endast mot en bråkdel av de olika varianter av pneumokocker som cirkulerar. Dessutom har fattiga länder, där flest barn dör, begränsad tillgång till pneumokockvaccin i dagsläget.

Pneumokocker finns emellertid periodvis i näsan hos en stor andel barn i världen utan att barnen blir sjuka, vilket kan tyckas paradoxalt. I dessa fall är barnen **bärare** av pneumokocker. Det är först när pneumokockerna sprider sig från de övre luftvägarna till andra delar av kroppen som de orsakar sjukdom, t.ex. öroninflammation som är en vanlig sjukdom hos barn. Ett mindre antal barn drabbas av mer allvarliga sjukdomar, såsom lunginflammation, hjärnhinneinflammation eller blodförgiftning. Dessa sjukdomar förekommer hos människor i alla åldrar, men det är främst de yngsta och äldsta individerna som är utsatta.

Hur kommer det sig att en och samma bakterie kan å ena sidan leva i **symbios** med människan och å andra sidan orsaka allvarliga sjukdomar? Man kan tänka sig att både immunförsvaret hos den infekterade personen och faktorer hos bakterierna spelar en roll. Min forskning har syftat till att skapa en bättre förståelse för samspelet mellan pneumokocker och det mänskliga immunförsvaret för att möjliggöra utvecklingen av nya typer av vaccin och behandlingsstrategier.

Det finns celler som är specialiserade på att aktivera och dirigera immunförsvaret så att sjukdomsalstrande bakterier snabbt kan upptäckas och elimineras. De kallas **dendritiska celler**. En detaljerad förståelse för vad som händer när dendritiska celler möter pneumokocker saknas och jag har därför studerat olika aspekter av samspelet mellan denna celltyp och pneumokocker. I min forskning har jag kunnat visa att flera viktiga funktioner hos de dendritiska cellerna slås ut av pneumokocker. Denna bakterie producerar nämligen ett gift, **pneumolysin**, som gör att de dendritiska cellernas förmåga att kommunicera med andra celler i immunförsvaret försvagas. Detta kan vara ett sätt för pneumokockerna att undvika att bli upptäckta av immunförsvaret.

Man vet att andelen bärare är lägre bland äldre barn, och hos vuxna är andelen bärare mycket låg. Vad har immunförsvaret för roll i denna process? Har äldre barn och vuxna bättre motståndskraft mot pneumokocker? Forskare har studerat hur sådan motståndskraft skulle kunna uppstå och det har visat sig att **T-hjälparceller** är viktiga i sammanhanget. Av denna anledning har jag studerat hur dendritiska celler dirigerar T-hjälparceller i försvaret mot pneumokocker. Jag har kunnat visa att **peptidoglykan**, som är en del av pneumokockens cellvägg, aktiverar de dendritiska cellerna. Detta leder i sin tur till att T-hjälparcellerna startar en **inflammation**, vilket är det symtom som dominerar hos patienter med pneumokocksjukdomar.

Nyligen har forskare upptäckt att **D-vitamin** är viktigt för vårt immunförsvar och jag har därför också velat undersöka hur dendritiska celler påverkas av D-vitamin. Mina resultat tyder på att tillsats av D-vitamin dämpar den inflammatoriska process som pneumokockerna triggar. Eftersom pneumokockinfektioner ofta leder till en okontrollerad inflammation som skadar kroppens vävnader så kan man tänka sig att D-vitamin skulle kunna ha terapeutisk potential. Mer forskning inom ämnet är emellertid nödvändig för att tydligare förstå dessa mekanismer.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Littmann M**, Albiger B, Frentzen A, Normark S, Henriques-Normark B, Plant L.
Streptococcus pneumoniae evades human dendritic cell surveillance by pneumolysin expression
EMBO Molecular Medicine 1, 211-222. 2009.

II. **Olliver M**, Hiew J, Mellroth P, Henriques-Normark B*, Bergman P*.
Human monocytes promote Th1 and Th17 responses to *Streptococcus pneumoniae*
Infection and Immunity 79, 4210-4217. 2011.

III. **Olliver M**, Hiew J, Bergman P*, Henriques-Normark B*.
Human dendritic cells, activated by pneumococcal peptidoglycan, promote innate and adaptive immune responses which can be modulated by vitamin D
Manuscript.

IV. Cederlund A, **Olliver M**, Rekha R, Lindh M, Lindbom L, Normark S, Henriques-Normark B, Andersson J, Agerberth B, Bergman P.
Impaired release of antimicrobial peptides into nasal fluid of Hyper IgE and CVID patients
PLoS One 6, e29316. 2011.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AMP	Antimicrobial peptide
APC	Antigen presenting cell
CAP	Community-acquired pneumonia
CBP	Choline binding protein
CFU	Colony-forming unit
CVID	Common variable immunodeficiency
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
HIES	Hyper-IgE syndrome
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
MAMP	Microbe associated molecular pattern
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NOD	Nucleotide-binding oligomerization domain
PBMC	Peripheral blood mononuclear cell
p.i.	Post infection
PID	Primary immunodeficiency
PRR	Pattern recognition receptor
RTI	Respiratory tract infection
STAT3	Signal transducer and activator of transcription 3
TA	Teichoic acid
TCR	T cell receptor
Th cell	T helper cell
TLR	Toll-like receptor
VDR	Vitamin D receptor

CONTENTS

PREFACE

Streptococcus pneumoniae (the pneumococcus) is one of the foremost bacterial pathogens in humans. Paradoxically, it is essentially a commensal organism that asymptomatically colonizes the nasopharynx of a significant proportion of the human population. Hence, a key question is why the majority of colonized individuals do not develop disease. Part of the answer lies in the dynamic interplay between pneumococci and cells of our immune system. To study the complex interactions between bacteria and immune cells has been an intriguing challenge, and my hope is that the work presented in this thesis provides insights into human immunity to the pneumococcus that can be used to identify possible points of intervention for treatment or vaccination.

1 INTRODUCTION

1.1 *STREPTOCOCCUS PNEUMONIAE*

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive encapsulated bacterium that was independently isolated and described by Louis Pasteur and George Sternberg in 1881 [2, 3]. It is a facultative anaerobe that can be grown on blood agar plates where it forms macroscopic colonies, and in the laboratory it can be identified by its α-hemolytic activity and optochin sensitivity. A thick polysaccharide capsule surrounds the pneumococcus (**Figure 1**), and based on differences in the composition of this capsule, more than 90 pneumococcal serotypes can be distinguished.

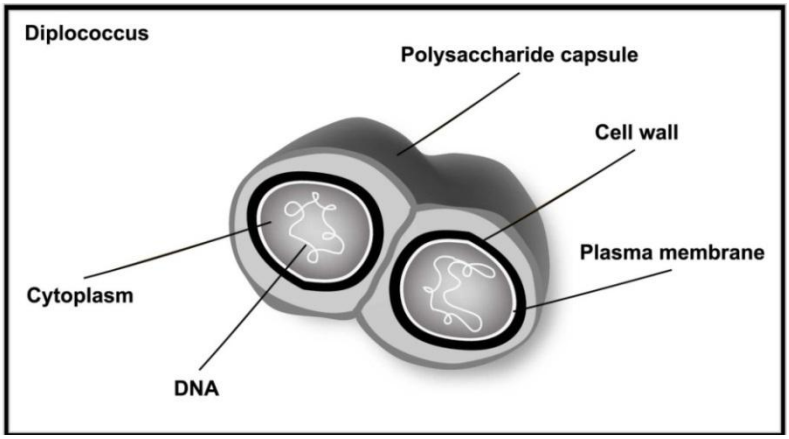

Figure 1. ***Streptococcus pneumoniae*.** In the microscope, pneumococci appear as oval-shaped single cells, diplococci or chains. © M. Olliver

The pneumococcal genome is a closed, circular DNA structure that contains approximately 2 million base pairs. Analysis of an increasing number of whole genome sequences has revealed that the pneumococcus is a genetically diverse bacterium. In fact, the genetic information can vary up to 10% between strains, and this is due to the fact that the pneumococcus is naturally transformable, i.e. it takes up DNA from the surroundings. This phenomenon was discovered by Frederick Griffith in 1928. He demonstrated that an unencapsulated avirulent pneumococcal strain could become encapsulated and virulent after the addition of a "transforming factor" from a heat-killed encapsulated strain [4]. In 1944, a time when it was widely believed that protein was the hereditary material of bacteria, Theodor Avery and colleagues made the groundbreaking discovery that the "transforming factor" in Griffith's experiment was DNA [5].

1.1.1 The Pneumococcus – A Commensal or Pathogen?

The pneumococcus is a human-specific bacterium. Hence, it has no animal reservoir and is not present in the environment. Instead, the main ecological niche for pneumococci is the human nasopharynx, where it forms part of the normal flora.

Asymptomatic nasopharyngeal carriage is most prevalent in young children, whereas adults are seldom carriers. When nasopharyngeal cultures for 1300 adults and 404 children were analyzed, *S. pneumoniae* was carried by 53% of children (≤ 6 years), compared with 4% of the adults in the same community [6]. In children attending day care centers, up to 60% harbor the pneumococcus in the nasopharynx [7, 8].

Although transient nasopharyngeal colonization rather than disease is the normal outcome of exposure to *S. pneumoniae*, it is nevertheless a major human pathogen, causing substantial morbidity and mortality worldwide. Disease occurs when bacteria are inhaled into the alveoli, enter the bloodstream, or cross the blood-brain barrier, causing pneumonia, sepsis or meningitis. This versatile bacterium can also cause more benign conditions such as otitis media and sinusitis.

Certain individuals have an increased risk of acquiring pneumococcal infections. Age, genetic background, socioeconomic status, immune status, geographic location and underlying disease are among the factors that determine the incidence of pneumococcal disease (**Table 1**).

Table 1. Examples of risk factors for pneumococcal disease.

Risk factor	Examples
Underlying disease	Heart, kidney, liver or lung disease, cancer, diabetes [9, 10]
Extremes of age	<2 years or ≥65 years
Primary immunodeficiency	Sickle cell disease, common variable immunodeficiency, agammaglobulinemia, Hyper-IgE syndrome [11-14]
Acquired immunodeficiency	Organ transplant recipients, HIV infection [15]
Crowding	Schools, day care centers, jails, hospitals, military camps [16, 17]
Ethnicity	Indigenous peoples of Alaska, Australian aborigines, and New Zealand Maoris [18-20]
Living in a developing country	Poverty, malnutrition, poor access to medical care [1]
Previous infection	Influenza virus [21, 22]
Other	Alcohol and drug abuse, exposure to cigarette smoke [23]

So is the pneumococcus a commensal or a pathogen? This question does not have a simple answer. Episodes of nasopharyngeal colonization usually do not lead to disease; hence, the pneumococcus is considered a commensal organism. When the host is in equilibrium with its commensal population of pneumococci, the bacteria are asymptomatically carried in the nasopharynx, which helps them persist in the human population. Here they can pass unnoticed while they live and feed on their host. However, in certain cases, host immune mechanisms are insufficient or dysregulated, rendering the host more susceptible to pneumococcal disease. This kind of imbalance may lead to immune-mediated pathology, since the pneumococcus is capable of triggering a highly inflammatory response that can become independent of bacterial

presence and lead to multiple organ failure and death. Thus, if this fine balance is broken, the pneumococcus has the potential to become a pathogen.

1.1.2 Global Burden of Pneumococcal Disease

Data on the global burden of pneumococcal disease are still limited; however, it has been estimated that pneumococcal disease annually cause about 826,000 deaths in children under the age of five [1] Even this is probably an underestimate since surveillance in high-mortality developing countries usually under-detects bacterial meningitis and sepsis incidence due to the low sensitivity of diagnostic tests and the limited access to care. The highest mortality rates are found in sub-Saharan Africa and south Asia (**Figure 2**), where ten countries account for 61% of all pneumococcal deaths [1].

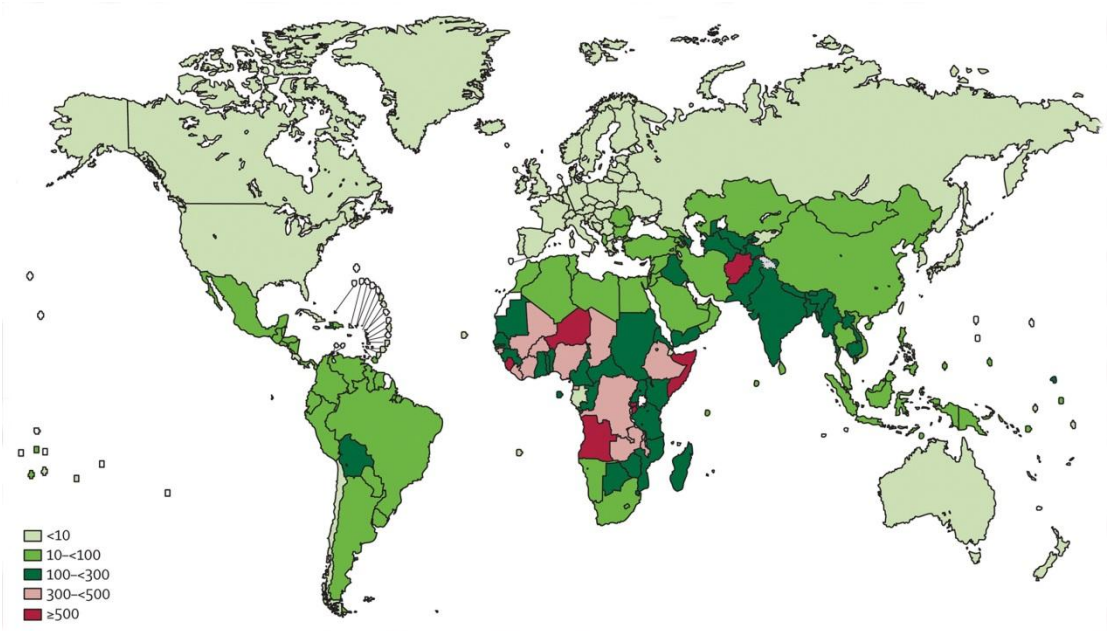

Figure 2. Pneumococcal mortality rate. Pneumococcal deaths in children aged < 5 years per 100 000 (HIV-negative pneumococcal deaths only). Adapted from [1].

Population-based data on invasive pneumococcal disease (IPD) in developed countries suggest an annual incidence of at least 15-20 cases per 100,000 persons of all ages and at least 50 cases per 100,000 elderly adults (≥65 years) [24]. These estimates correspond with Swedish statistics from 2011, where the incidence of IPD was 14 cases per 100,000 persons of all ages, with 66% of cases in adults older than 60 years [25].

Pneumococcal serotypes differ in carriage prevalence and disease incidence [26], and the spectrum of prevailing capsular types varies with age, time and geographical region. There have been numerous epidemiological studies to investigate which serotypes are the most prevalent causes of disease in certain geographical regions at certain time points. These types of studies show that the majority of the burden of pneumococcal disease is associated with a rather restricted number of serotypes [27].

Moreover, it appears that some serotypes mainly affect younger children while others mainly affect the elderly [28]. Furthermore, some serotypes mainly infect previously healthy individuals whereas others primarily affect patients with underlying disease [28], suggesting that some pneumococcal isolates behave as primary pathogens whereas others are more opportunistic.

1.1.3 Prevention and Treatment of Pneumococcal Infections

The fight against pneumococcal disease has achieved significant results in the last decades, but the challenge is still ongoing. The main strategy to prevent pneumococcal infections is through vaccination, and the development of effective and affordable vaccines has been a global health priority for many decades. However, there are several challenges for vaccine development, as will be discussed here.

Prevention

Two vaccine formulations are available to prevent pneumococcal infection: the *polysaccharide* vaccine and the *conjugate* vaccine. The polysaccharide vaccine is based on capsule components of the 23 most common capsular serotypes that cause severe pneumococcal disease in the developed world. This type of vaccine has been in use since the 1970s [29] and is recommended for individuals older than 65 years, as well as other groups with an increased risk of pneumococcal disease.

Antibodies to the capsular polysaccharide antigens provide serotype-specific protection against pneumococcal infections. However, this vaccine induces a T-cell-independent B cell response, and therefore its effectiveness is hampered by poor responses in children younger than 2 years of age who have inadequate production of antibodies to polysaccharide antigens. For this reason, polysaccharide vaccines fail to protect infants and small children [30]. There is also skepticism about the effectiveness and efficacy of this vaccine against pneumonia in the elderly and in immunocompromised adults [31].

Since young children and the elderly are precisely the risk groups for severe pneumococcal disease, several vaccine manufacturers have in the last 15 years developed new vaccines, in which a number of capsular polysaccharides are covalently coupled to a protein carrier, called conjugate vaccines. These vaccines are highly immunogenic in all age groups and induce a T-cell dependent immune response characterized by immune memory. Importantly, they stimulate mucosal immunity, which reduces nasopharyngeal carriage. Lower carriage rates among vaccinated children, who are thought to be the most important vector for horizontal dissemination of pneumococci [32], reduces transmission of vaccine-type pneumococci in the community, hence, conferring herd immunity among unvaccinated adults [33, 34].

A 7-valent conjugated pneumococcal vaccine was approved by the United States Food and Drug Administration (FDA) in 2000. This vaccine contains polysaccharides of the most seven common serotypes known at the time to cause IPD in the United States. 10- and 13-valent conjugate vaccines have now been introduced into routine infant immunization programs of several countries, including Sweden.

Use of the conjugate vaccine has modified the epidemiology of IPD in the population and led to a significant decrease in IPD with included serotypes [33, 35, 36]. Between 1999 and 2003 in the United States, the effectiveness of the 7-valent conjugate vaccine against IPD caused by vaccine serotypes in children below 5 years of age declined from 80 to 4.6 cases per 100,000 inhabitants, meaning an overall 94% decline [37]. In South Africa, vaccine efficacy against vaccine serotypes was 83% [38]. Reduction in pneumococcal disease has also been observed among unvaccinated age groups [39, 40], demonstrating that the conjugate vaccine provides herd immunity. However, despite the effectiveness of this vaccine, there may be a limited lifespan to the observed benefits, for the following reasons:

Coverage is limited

Although the conjugate vaccine has been shown to be effective against both invasive and non-invasive disease, the current licensed products contain a limited number of serotypes and cannot be expected to provide protection against carriage or disease caused by most other serotypes. In addition, coverage varies in different populations and may be lower in many developing countries. Furthermore, the prevalence of pneumococcal serotypes fluctuates over time and region [41], demonstrating the difficulty in choosing a limited number of capsular types to include in a polysaccharide-based vaccine

Expensive vaccine that does not reach the right population

An additional challenge in the prevention of IPD is for the vaccine to reach the right population. Despite the considerable burden of disease caused by pneumococci, the available conjugate vaccine has yet to be launched in the majority of low-income settings where it is most needed. Vaccination is urgently needed in Africa and Asia, which together account for 95% of all pneumococcal deaths [1]. One obvious limiting factor is the high price of the conjugate vaccine. Another factor is the cold chain, i.e. the vaccine requires a temperature of approximately 8 degrees Celsius, and thus transport and storage of the vaccine within the safe temperature range is essential.

Emergence of non-vaccine serotypes

In parallel with the dramatic decrease of IPD caused by vaccine serotypes, there has been an increased rate of IPD due to non-vaccine serotypes [42-44]. Evidence from several countries suggests that serotype replacement in carriage has emerged as a consequence of vaccination. Serotype 19A has become particularly important because an increased incidence of IPD caused by 19A has been shown in different populations [45, 46]. Mera et al. reported an increase in serotype 19A from 3% to 20% before and

after introduction of the vaccine [47]. Hence, continued surveillance of serotype distribution is essential to allow rational vaccine design.

These important shortcomings underline the need for developing improved pneumococcal vaccines in order to provide protective immunity against a larger number of serotypes. New vaccine strategies focus on the use of highly conserved immunogenic surface-associated proteins (reviewed in [48]). This type of vaccine has several advantages as it is expected to elicit protection in all age group, induce broad and serotype-independent protection, and be cheap to produce. Many protein vaccine candidates are under investigation, however, at present, the search for a protein-based universal pneumococcal vaccine covering all serotypes remains in its infancy.

Another possible approach, which has been evaluated by the group of Richard Malley, is a killed unencapsulated whole-cell vaccine, shown to be effective in preventing nasopharyngeal colonization with encapsulated pneumococci in mice [49]. This type of vaccine has properties that makes it suitable for use in developing countries, including coverage irrespective of serotype, low cost, stability following lyophilization (thus avoiding cold chain issues), and mucosal administration [50].

Although it may seem a good idea to completely eradicate pneumococci from the nasopharynx, there is an important caveat. Clearing the nasopharynx of this organism might lead to replacement with other species, providing opportunities for potential pathogens that normally compete with the pneumococcus for the nasopharyngeal niche. For this reason, perhaps one should hesitate before intervening with a bacterium that has been colonizing humans for a very long time. Also, acquisition of natural immunity to the pneumococcus might be more important than we realize, as multiple exposure events throughout life may maintain protection against pneumococcal disease. Ultimately, future pneumococcal vaccines should aim to mimic natural immunity with the objective to protect against disease rather than colonization.

Treatment

In the beginning of the 20th century, pneumococcal pneumonia was treated with type-specific antisera [51]. Controlled clinical trials conducted in the 1920s demonstrated the benefit of pneumococcal type-specific antiserum by comparing mortality among patients given antiserum treatment with that among control patients who received no specific therapy [52]. Rapid provision of serum to patients with pneumococcal pneumonia was a major public health initiative of the late 1930s. However, with the advent of antibiotics, the antiserum programs soon collapsed and pneumonia reverted to the domain of the private practitioner [53].

Penicillin, introduced in the 1940s, proved to be highly effective against pneumococci and has ever since been the most commonly used drug for pneumococcal infections. However, treatment of pneumococcal infections with β-lactam antibiotics, such as

penicillin, can result in the paradoxical enhancement of inflammation. This is due to the release of proinflammatory cell wall products [54], and it has been suggested that anti-inflammatory drugs should be administered concurrently with the antibiotics in patients with meningitis [55].

As with many other bacteria, the use of antibiotics has led to the emergence and spread of resistant strains of pneumococci. Resistance to penicillin is related to structurally modified penicillin-binding proteins that allow peptidoglycan synthesis despite the presence of penicillin. Penicillin resistant strains were first noted in the mid 1970s and resistant clones have now spread worldwide [56-58]. When susceptibility testing was examined in eight European countries, an overall of 25% of isolates were nonsusceptible to penicillin [59]. However, the prevalence of penicillin resistance varies between European countries, and in Sweden, resistance to penicillin among invasive isolates has remained around 3% [60].

Antimicrobial resistance of pneumococci to fluoroquinolones, macrolides, vancomycin and other antibiotics is increasingly recognized worldwide [61, 62]. Therefore, prudent use of antimicrobial agents is needed, as well as discovery of new antimicrobial agents. The escalation of antimicrobial resistance among pneumococci emphasizes the importance of preventing pneumococcal disease through immunization and the urgent need for development of new vaccines.

1.1.4 Disease Pathogenesis

Pneumococci are transmitted from person to person by sneezing, coughing or through direct contact. Colonization of the nasopharynx, especially in young children, provides the major reservoir for transmission of pneumococci [63], and nasopharyngeal carriage is thought to be a prerequisite of disease. Through a combination of virulence factor activity, the pneumococcus is able to evade the host immune response and spread from the nasopharynx to the ear, sinus, lung, blood and meninges, causing a variety of diseases (**Table 2**). When pneumococci invade normally sterile sites, such as the bloodstream and meninges, the resulting forms of pneumococcal disease are classified as invasive.

Table 2. Overview of diseases caused by pneumococci.

	Disease	Part of the body	Symptoms
Non-invasive	Otitis media	Middle ear	Pain in the ear and fever
	Sinusitis	Paranasal sinuses	Headache, pressure or pain in the sinuses, thick nasal discharge
	Pneumonia	Lung	Fever, productive cough, headache, shortness of breath, chest pains
Invasive	Sepsis	Blood	Fever, headache, muscular aches
	Meningitis	Meninges	Fever, headache, vomiting, neck stiffness

Otitis media

Acute otitis media is the most common clinical manifestation of pneumococci and at the age of two years, most children have experienced at least one episode of pneumococcal otitis media [64]. The pneumococcus is the leading cause of otitis media, although it can also be caused by other bacterial pathogens, including *Haemophilus influenzae* and *Moraxella catarrhalis*. Major risk factors for developing otitis media is eustachian tube dysfunction (ineffective clearing of bacteria from the middle ear) and repeated exposure to large numbers of other children, whether at home or in day care [64].

Very little is known about the process of which pneumococci gain access to the middle ear. The bacteria must first progress up the eustachian tube, and once in the middle ear, they trigger a cytokine response that results in the influx of neutrophils. Several animal studies have demonstrated that pneumococcal cell wall components play a major role in generating the inflammation that characterizes pneumococcal otitis media [65, 66]. Complications of otitis media include permanent hearing loss, mastoiditis (spread of the infection into the area of bone underneath the ear), facial paralysis and meningitis. Children with recurrent episodes of acute otitis media have a higher risk of developing hearing loss [67].

Sinusitis

The pneumococcus is one of the most common etiological agents of sinusitis, which is characterized by inflammation of the lining of the paranasal sinuses. It can be precipitated by allergy or a viral upper respiratory tract infection.

Pneumonia

S. pneumoniae is the most common cause of community-acquired pneumonia (CAP) [10]. In both Europe and the USA, CAP is the most frequent cause of death from infection [68]. Despite advances in diagnostic methods and intensive care support, mortality in pneumococcal pneumonia remains high, ranging from 7% to 36% [69, 70] and may exceed 50% in groups with predisposing factors [71]. In 20-30 % of cases with pneumococcal pneumonia, bacteria spread to the blood and cause sepsis. Pneumonia can also lead to complications such as pleural empyema (the accumulation of pus in the pleural cavity). After the introduction of the 7-valent conjugate vaccine, increases in the incidence of empyema, associated with the emergence of nonvaccine serotype 1, have been reported [72].

Pneumonia arises when pneumococci reach the alveoli where they multiply and spread, causing cytokine production and influx of white blood cells to the alveoli. The resulting tissue destruction leads to lack of oxygen and cyanosis. Alveolar macrophages

represent the first phagocytic defense in the lungs [73], however, when a large number of pneumococci are introduced into the lower airways, neutrophils become the main phagocytic cells in response to pneumococcal pulmonary infection in mice [74]. Migration of neutrophils into the lung tissue can be both good and bad; Neutrophils are considered important effector cells in the host defense but they can also induce inflammation and tissue damage. Accordingly, it has been found that depletion of neutrophils can either result in increased mortality [75] or improved survival [76] in intranasally infected mice. These findings suggest that the role played by neutrophils in pneumococcal pneumonia requires closer scrutiny. Interestingly, peripheral neutropenia is generally not considered to be a risk factor for adult pneumococcal disease. Nevertheless, neutropenia is a poor prognostic finding in patients with established pneumococcal disease [77].

Sepsis

When bacteria reach the bloodstream they can cause a systemic inflammation called sepsis, which, if not treated, can lead to multisystem organ failure and death. Pneumococcal sepsis is associated with a significant mortality rate, ranging from 13% to 36% [78-80] and at least 50% of mortality occurs within the first 48 hours of admission [78, 79].

The mechanisms by which pneumococci breach the epithelium and get access to the circulation are still not well understood. Bacterial cell wall fragments may stimulate the release of cytokines and chemokines, and mechanisms that normally control a local infection become detrimental during this systemic dissemination. From the blood, pneumococci may pass the blood-brain barrier and cause meningitis.

Meningitis

The most common etiological agents of community-acquired bacterial meningitis are *S. pneumoniae* and *Neisseria meningitidis*. Despite advances in medical care, mortality from pneumococcal meningitis ranges from 25-30% [81-83]. Neurological sequelae are common and include hearing loss, cognitive impairment, blindness, and paralysis. Neuronal damage in bacterial meningitis is caused by the dual effects of an overwhelming inflammatory reaction and direct effects of bacterial toxins [84]. Pneumococcal pneumolysin and hydrogen peroxide (H_2O_2) have been shown to mediate brain cell apoptosis in mice [85]. The same investigators showed that pneumolysin functions as a mitochondrial toxin and a determinant of caspase-independent apoptosis, causing neuronal cell death [86].

1.2 THE IMMUNE SYSTEM

Our immune system is an extremely complex organization of multiple cell types and structures that together make up a defense system against microorganisms. The immune system can be divided into two categories: the *innate* and the *adaptive* host defense. The innate immune system rapidly recognizes bacterial, viral, fungal and parasitic elements that are conserved across organisms, whereas adaptive immune responses develop against specific epitopes contained within these organisms. However, it is important to note that the innate and adaptive immune systems work together in the clearance of microbes.

1.2.1 Innate Immunity

The innate immune system includes cells such as monocytes, macrophages, dendritic cells (DCs), mast cells, NK cells and neutrophils. There are also humoral components such as the complement system and antimicrobial peptides (AMPs). Additionally, innate immunity includes anatomical barriers such as the skin and epithelium, as well as secreted mucus. Innate host responses are critical to the outcome in bacterial infections and will be discussed here in relation to pneumococcal infections.

Dendritic cells

DCs are crucial cells of the innate immune system, with a superior ability to take up, process and present antigens compared with other antigen presenting cells (APCs). They are often described as the "conductors" of the immune response in their capacity to orchestrate signals derived from different parts of the immune system, serving as a link between innate and adaptive immunity [87]. DCs are particularly abundant at the major portals of microbial entry, including skin and mucosa [88], and in the lungs, they form a network of sentinel cells specialized in sampling inhaled bacterial pathogens [89, 90].

Ralph Steinman and Zanvil Cohn were the first to describe dendritic cells [91]. They identified this cell in the secondary lymphoid organs of mice using microscopy techniques. The most striking feature of the DC is its surface projections, which extend and retract from the cell body. In 2011, Ralph Steinman was awarded the Nobel Prize in Physiology or Medicine for his discovery of the DC and its role in adaptive immunity [92].

DCs can be categorized into multiple subsets. The two broadest categories are myeloid and plasmacytoid DCs. Myeloid DCs include epidermal and dermal DCs, which are found in peripheral tissues, as well as immature myeloid DCs that circulate in the blood. Myeloid DCs can also be generated *in vitro* from monocyte blood precursors.

Plasmacytoid DCs are found in blood as well as in peripheral lymphoid organs, and can produce large amounts of IFN-α and IFN-β upon stimulation.

Maturation of DCs is a pivotal process for initiating immunity (**Figure 3**). On recognition of microbes, DCs undergo a process of maturation which is characterized by the upregulation of MHC, as well as costimulatory molecules CD80 and CD86 [93]. Maturation also results in the expression of cytokines and chemokines, and increased antigen processing and presentation. Mature DCs are able to prime naïve T cells by the release of cytokines that promote T helper (Th) 1, Th2, Th17 or regulatory T cells.

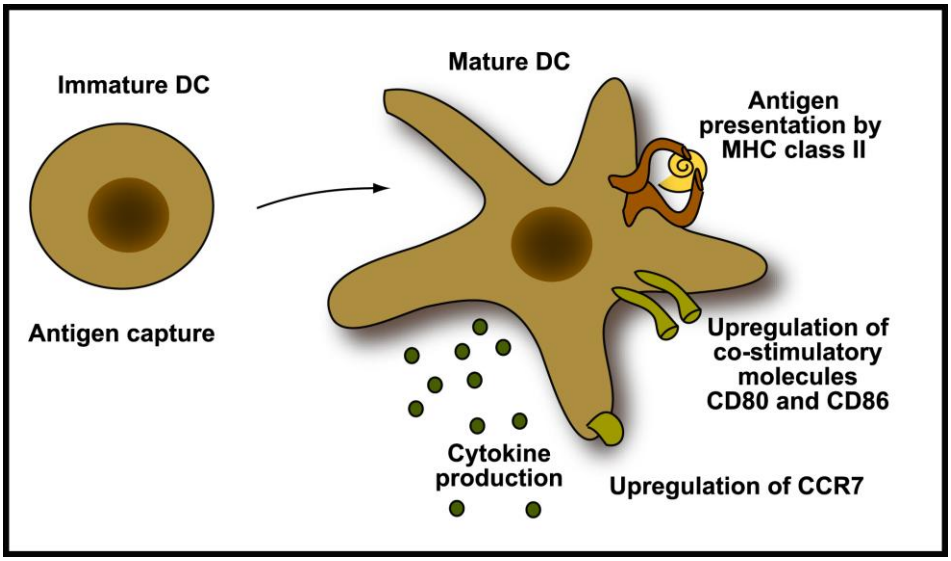

Figure 3. DC maturation. Immature DCs lose their phagocytic capacity and up-regulate MHC class II, the co-stimulatory molecules CD80 and CD86, and the chemokine receptor CCR7. They also start producing cytokines and chemokines. ©

One of the hallmarks of DC biology is their ability to migrate. Following antigen uptake in tissues, DCs migrate to the draining lymph nodes to stimulate T cell responses. Hence, expression of the lymphoid chemokine receptor CCR7 is also induced during DC maturation [94]

To promote colonization or cause invasive disease pneumococci have to overcome DC-based immunosurveillance in the upper respiratory tract. Cytokines and chemokines produced by the DCs at the site of pneumococcal entry will drive inflammatory signals which regulate resident and newly arrived phagocytes. DC-produced cytokines will also play a role in the effector functions of B and T cells. However, the mechanisms of recognition, uptake and intracellular fate of intact pneumococci by DCs have not been studied in great detail. One of the objectives with this thesis was thus to characterize DC responses to live pneumococci and identify bacterial factors involved in cellular activation. These kinds of experiments can provide basic information about the pathogenesis of pneumococcal infections, giving us further insight into the immune modulation induced by pneumococci, and improve the likelihood that we can manipulate these responses for our own interest.

Monocytes, Macrophages and Neutrophils

Phagocytes are immune cells that can ingest (phagocytose) particles, such as bacteria, parasites and dead host cells. Besides DCs, the professional phagocytes of the human immune system include monocytes, macrophages and neutrophils. Phagocytes have many types of receptors on their surface that increase phagocytosis of bacteria which have been coated with antibodies or complement.
Monocytes participate in innate immune responses through the effector and regulatory functions of monocyte-derived macrophages and DCs. Interestingly, Randolph et al. showed that monocytes are able to migrate from peripheral tissues into the T cell zone of draining lymph nodes upon stimulation [95]. Hence, activated monocytes may be involved in T cell differentiation *in vivo*.

Macrophages are derived from blood monocytes and are present in virtually all tissue. Alveolar macrophages, a type of macrophage found in the pulmonary alveolus, are considered major effector cells in host defense against respiratory tract infections by virtue of their potent phagocytic properties. In murine models of pneumococcal pneumonia it has been shown that following exposure to pneumococci, alveolar macrophages rapidly transport bacteria from the lung to draining lymph nodes [96]. Alveolar macrophages also have a protective anti-inflammatory role, possibly by clearing apoptotic neutrophils [97].

Neutrophils are among the first immune cells recruited from the blood stream to the site of infection. They recognize bacteria via C3b complement receptor (CR3) or Ig Fc receptors and engulf them into vesicles called phagosomes. Bacteria are killed with non-oxidative (AMP-mediated) and oxidative mechanisms when the phagosome fuses with intracellular granules to form a phagolysosome. Neutrophils can form neutrophil extracellular traps (NETs), which are networks of extracellular fibers, composed of chromatin DNA, histones, enzymes and AMPs. NETs can kill microbes exracellularly by providing a high local concentration of antimicrobial components. Beiter et al. found that NETs are formed in the lungs of mice intranasally infected with pneumococci, and that pneumococci are captured but not killed by NETs *in vitro* [98]. Expression of the DNase EndA and the polysaccharide capsule, were identified as factors that helped pneumococci evade NETs [98].

Pattern Recognition Receptors

The first recognition of bacteria by the host is mediated by pattern recognition receptors (PRRs). These receptors are activated by microbe-associated molecular patterns (MAMPs), bacterial virulence factors, as well as endogenous molecules released after tissue damage. Activated PRRs regulate the production of inflammatory cytokines that, in turn, stimulate neighboring immune and non-immune cells, starting an immune response.

Examples of PRRs are the Toll-like receptors (TLRs) and the NOD-like receptors (NRLs), which will be described here. A combination of signaling through TLRs and NLRs leads to the synergistic activation of immune responses, and a crosstalk between these receptors is probably crucial for the balance of the immune effector arms.

Toll-like receptors

TLRs are a family of host defense receptors that consists of 10 members in humans (**Table 3**) and 13 in mice. APCs have been the primary focus of TLR investigation; however, TLRs are also expressed on cells of the adaptive immune system [99] and non-immune cells, such as epithelial cells [100]. TLRs found in the epithelium of the respiratory tract may thus contribute to the immune response to airborne antigens such as *S. pneumoniae*.

Table 3. TLRs and their ligands.

Receptor	Location	Ligand
TLR1/2	Cell surface	Lipoproteins
TLR2	Cell surface	Lipoproteins, Lipoteichoic acid, Zymosan
TLR2/6	Cell surface	Di-acyl lipopeptides
TLR3	Endosome	Double-stranded viral RNA
TLR4	Cell surface	LPS from Gram negative bacteria Pneumolysin
TLR5	Cell surface	Bacterial flagellin
TLR7	Endosome	Single-stranded viral RNA
TLR8	Endosome	Single-stranded viral RNA
TLR9	Endosome	Bacterial CpG motifs
TLR10	Cell surface	Unknown

Several TLRs have been shown to play important but partly redundant roles in the innate defense against pneumococci (reviewed in [101]). TLR2 has been investigated in different mouse models and is suggested to enhance pneumococcal phagocytosis and intracellular killing [102, 103], and protect the host during meningitis [104]. However, other investigators have established that TLR2 does not contribute to an effective antibacterial defense [105, 106], suggesting that other components of the immune system are sufficient to maintain an adequate response.

TLR9 has been implicated as an important player in protective immunity in pneumococcal pneumonia. $TLR9^{-/-}$ mice had reduced survival in a model of pneumonia, and macrophages deficient in TLR9 were shown to be impaired in pneumococcal uptake and killing [106].

In addition to TLR2 and TLR9, TLR4 appears to play a role in pneumococcal infection by its sensing of pneumolysin [107]. However, it is possible that this is an indirect effect of TLR4 recognition of endogenous ligands released after bacteria-induced host cell damage [108].

Nod-like receptors

The family of NLRs consists of 22 mostly cytosolic proteins in humans and 33 members in mice. Nucleotide-binding Oligomerization Domain (NOD) 1 and 2 are cytosolic receptors that have important roles in innate immunity as sensors of microbial components derived from bacterial peptidoglycan. NOD1 detects peptidoglycan fragments produced by Gram-negative bacteria, whereas NOD2 is activated by peptidoglycans of basically all bacteria [109]. Specifically, NOD2 is a sensor of peptidoglycan through the recognition of muramyl dipeptide (MDP), the minimal bioactive peptidoglycan motif common to all bacteria [110].

Opitz et al. showed that NOD2 activates the transcription factor NF-κB after detecting internalized pneumococci *in vitro* [111] and Davis et al. recently showed that macrophage phagocytosis and digestion of pneumococci leads to pneumolysin-mediated delivery of peptidoglycan fragments into the host cell cytosol, triggering NOD2 activation [112].

Antimicrobial Peptides

AMPs are components of the innate immune response that have a broad spectrum of antimicrobial activity against viruses, bacteria and fungi [113]. The initial contact between AMPs and bacteria is thought to be electrostatic since AMPs are positively charged and bacterial surfaces are negatively charged. The majority of AMPs kill bacteria by inserting themselves into the cell membrane bilayers, forming pores that disrupt cell membrane function.

In humans, there are two main families of AMPs: *defensins* and *cathelicidins* [113]. The two main defensin subfamilies are α-defensins (produced by neutrophils and intestinal Paneth cells) and β-defensins (produced by epithelial cells of the lung, skin and gut). Defensins are abundantly represented in humans cells and tissues and because of their ability to kill a variety of microbes under laboratory conditions, they are thought to function as natural antibiotics [114].

Only one type of cathelicidin, called LL-37, is found in humans and is produced by neutrophils, mononuclear cells and epithelia. In addition to its antimicrobial effects, LL-37 has prominent chemotactic activities on neutrophils and T cells [115]. Interestingly, incubation of human DCs with LL-37 caused an increased activation of the DCs [116], suggesting that LL-37 released upon triggering of innate immunity may shape adaptive immune responses through an interaction with DCs.

1.2.2 Adaptive Immunity

The adaptive immune system is composed of highly specialized cells, including antibody-producing B cells and various kinds of Th cells and cytotoxic T cells. By

genetic recombination, B and T cells can generate a vast number of different antigen receptors, uniquely expressed on each individual cell. This process results in the generation of a diverse, yet specific, repertoire of immune effectors.

B Cells

The principal functions of B cells are to make antibodies, perform the role of APCs and develop into memory cells. Antibodies, also known as immunoglobulins (Igs), are large proteins that recognize and bind to antigens. There are five different classes of antibodies: IgG, IgM, IgA, IgE and IgD, which have different characteristics.

B cell immunity appears to be necessary for protective immunity against pneumococci, as patients with B cell defects are prone to develop pneumococcal disease [13]. Patients with IgG2 subclass deficiency are for example more prone to develop sinusitis, pneumonia and IPD [117]. Little information is available on IgA deficiency and pneumococcal disease. IgA deficiency has a high incidence (1/800) but only 30% of patients with a complete lack of IgA in serum (<0.07 g/L) exhibit an increased susceptibility to infections. The reason for this observation is still unknown. In addition, IgA-deficient patients have a greater risk of developing autoimmune diseases, such as type 1 diabetes and thyroid disease, suggesting an underlying dysregulation of the immune system. Given the high prevalence of IgA-deficiency and the incomplete penetrance of the clinical phenotype, it is difficult to know if pneumococcal disease among these patients is caused by the immune defect *per se* or is a mere coincidence.

T Cells

T cells only recognize antigen when it is presented by an APC in the context of an MHC-peptide complex. There are two subset of T cells; $CD8^+$ cytotoxic T cells and $CD4^+$ Th cells.

Naïve $CD4^+$ T cells differentiate into effector cells when they encounter a foreign antigen presented by an APC in the context of environmental factors. The Th1 and Th2 cell paradigm, which was first proposed by Mosmann and Coffman [118], has been used to explain how the host elicits different adaptive immune responses to eradicate various pathogens. Th1 effectors produce IFN-γ and regulate cellular immunity against intracellular infections, whereas Th2 cells produce IL-4, IL-5 and IL-13 and are important for humoral immunity and control of parasitic infections.

The traditional paradigm of Th1/Th2 cell dichotomy changed when a third subset of Th effector cell was described: Th17 cells [119, 120]. Upon stimulation, Th17 cells acquire the capacity to produce the inflammatory cytokines IL-17, IL-21 and IL-22. The cytokines that instruct Th17 cell lineage development likely include IL-6, IL-1β,

IL-23 and IL-21 [121, 122]. Cytokine-driven activation of signal transducer and activator of transcription 3 (*STAT3*) pathway is an essential step in Th17 cell differentiation [123]. Th17 cells initially developed a reputation as a destructive element in animal models of autoimmune disease [124]. In humans, the reputation was due to correlative data documenting an increase in IL-17-producing cells at sites of tissue inflammation. Hueber et al. found that human neutralizing antibodies to IL-17 are effective in both psoriasis and rheumatoid arthritis [125], indicating that Th17 cells are likely to be harmful in these diseases. However, we now know that although Th17 cells participate in the pathogenesis of several autoimmune diseases, they can contribute to protection in other diseases, by mediating a diverse set of responses. They are essential in the host defense against various bacterial, viral and fungal infections, and the responses elicited by Th17 cytokines are important for controlling dissemination of infectious agents beyond the mucosa [126, 127]. Interestingly, IL-17 is not only produced by Th17 cells; under certain conditions γδT cells [128, 129], $CD8^+$ T cells [130] NKT cells [131] can secrete IL-17.

The ability of IL-17 to mobilize neutrophils is believed to be the principal reason as to why this cytokine is important for protection against infectious pathogens [132]. IL-17 induces neutrophil influx through the production of cytokines and chemokines such as IL-6, IL-8 and GM-CSF [133]. In addition, the Th17 cytokines IL-17 and IL-22 cooperatively enhance expression of AMPs associated with host defense [134, 135] (**Figure 4**).

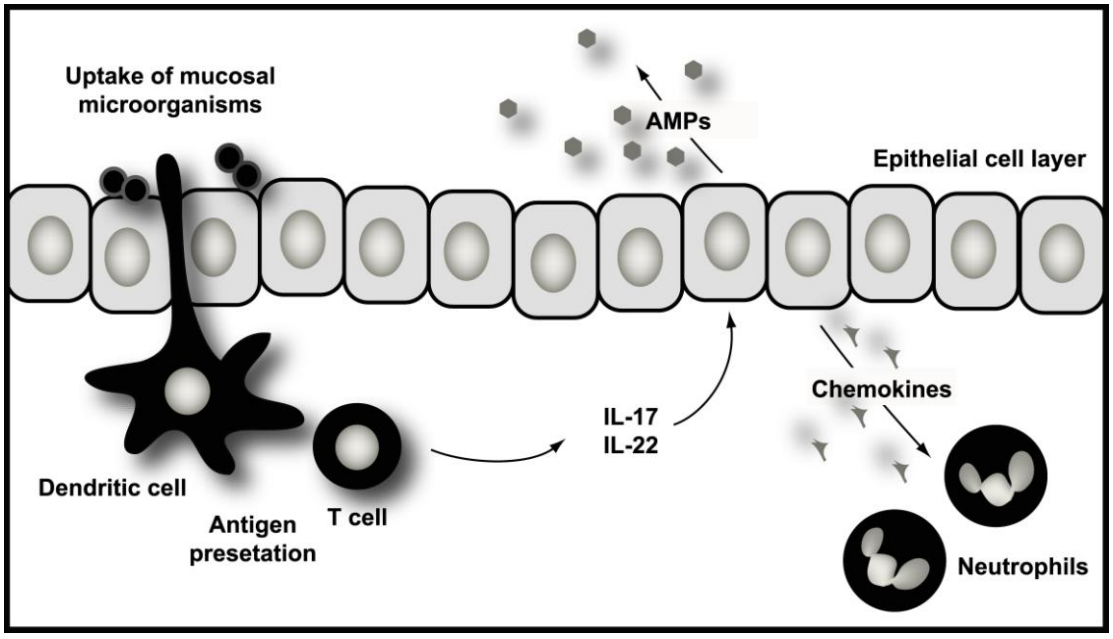

Figure 4. Role of Th17 cells in the mucosa. Uptake and presentation of mucosal microorganisms by DCs promote Th17 cell production of IL-17 and IL-22. These cytokines induce AMPs and neutrophil-recruiting chemokines by epithelial cells. © M. Olliver

Lu et al. demonstrated a critical role for IL-17 in mediating acquired immunity to pneumococci in a murine colonization model [136]. The same investigators also showed that IL-17 increases pneumococcal killing by human neutrophils, although the

precise mechanism involved was not identified. Th17 responses appear to be critical for immunity against colonizing pneumococci, as depletion of IL-17 or $CD4^+$ T cells blocked the recruitment of monocytes/macrophages and neutrophils, and diminished pneumococcal clearance [137]. In contrast, Th17 cells were not required in the rapidly invasive model of pneumonia described by Cohen et al. [138].

Despite recent advances, little is known about how pneumococci generate Th cell responses in the human host. In one recent study, Mureithi et al. characterized T cell memory responses to pneumococci in healthy adults in an area of high pneumococcal carriage and disease. They found that pneumococci triggered both Th1 (IFN-γ) and Th17 (IL-17) cytokine responses in peripheral blood mononuclear cells (PBMCs), but that the level of T cell memory was not associated with interruption of pneumococcal carriage [139]. Further studies of Th cell responses in humans are needed to characterize T cell pneumococcal immunity acquired through asymptomatic carriage.

1.2.3 Immunomodulatory Effects of Vitamin D

The classic function of vitamin D is to enhance intestinal absorption of calcium by regulating calcium transport proteins in the small intestine. In the absence of vitamin D, dietary calcium is not properly absorbed, resulting in hypocalcemia which can cause rickets (softening of bones) in children and adolescents. However, the importance of vitamin D in the regulation of cells of the immune system has gained increased appreciation over the past decade.

Humans obtain vitamin D precursors by exposure of their skin to the ultraviolet B (UVB) component of sunlight, and from diet, but to a much smaller extent. Activation of vitamin D requires two sequential hydroxylation steps. In the first step (in the liver) the enzyme 25-hydroxylase converts vitamin D to the inactive, circulating, form 25(OH)D3. In the second step (in the kidney, as well as other tissues), 25(OH)D3 is activated by the enzyme 1-alpha hydroxylase (Cyp27B1), yielding the active form of vitamin D; $1,25\text{-}(OH)_2D3$.

Responsiveness to vitamin D depends on expression of the nuclear vitamin D receptor (VDR) which binds to specific vitamin D response elements in the promoters of approximately 200 target genes in the human genome [140]. Nearly every tissue in the body has receptors for the active form of vitamin D. As reviewed by Di Rosa et al. [141], all immune cells express the VDR, including DCs and activated T cells [142-144]. Potent immunomodulatory activities of vitamin D on both innate and adaptive immune responses have recently been discovered (**Table 4**). Many studies suggest that vitamin D can enhance innate immunity while dampening overly zealous adaptive immune responses; hence, it appears to play an important role in maintaining immune homeostasis.

The anti-inflammatory role of vitamin D has been documented in various bacterial infections [145]. Based on results by Yim et al., [146] it has been speculated that the use of inhaled vitamin D could augment the expression of cathelicidin on the mucosal surface of bronchial epithelia, thereby increasing the antibacterial activity against airway pathogens in patients with cystic fibrosis.

Vitamin D deficiency is now recognized as a pandemic and it has been speculated that the alarming prevalence of vitamin D deficiency may be contributing to immune-mediated diseases. Very few foods naturally contain vitamin D, so sun exposure is the major source of vitamin D. As early as in the 1920s, sun exposure was recognized as an effective treatment for pulmonary tuberculosis but with the advent of antibiotics after the First World War, the idea that regular sun exposure could protect against infection was forgotten.

Table 4. Effects of vitamin D on the immune system

Cell type	Effect	Reference
Monocytes/ Macrophages	Induces autophagy via cathelicidin to facilitate destruction of *Mycobacterium tuberculosis* within auto-phagolysosomes	[147]
Monocytes/ Macrophages	Triggers antimicrobial activity	[148]
Monocytes	Inhibits differentiation into DCs *in vitro*	[149]
Bronchial epithelial cells	Increases antibacterial activity against airway pathogens by stimulating induction of cathelicidin	[146]
Monocyte-derived DCs	Increases IL-1β and IL-6 production and inhibits antigen presentation	[150]
$CD4^+$ T cells	Inhibits production of IFN-γ and IL-17	[151]
T cells	Induces regulatory T cell responses	[152]
Macrophages and epithelial cells	Induces AMPs	[153]

Due to the immunoregulatory and immunosuppressive roles of vitamin D, there has been increasing clinical interest for applications of vitamin D in immune-related disorders. Animal models with vitamin D have shown promising results in autoimmune diseases such as type 1 diabetes [154] and arthritis [155].

Martineau and colleagues undertook a randomized controlled trial of vitamin D in adults with pulmonary tuberculosis in 2011. They assessed the potential of 10 mg supplemental vitamin D to accelerate rates of sputum culture conversion, and found that a small subset of patients with the *tt Taql* VDR genotype had enhanced sputum culture conversion rates with vitamin D [156]. Thus, inherited factors may influence responses to vitamin D supplementation. In a similar study by Wejse et al., supplementary vitamin D did not improve clinical outcome and had no overall effect on mortality in tuberculosis patients [157]. However, in this study, the

supplementation group did not show increased serum 25(OH)D3 levels when compared with the placebo group, which makes it difficult to interpret the data.
In another recent clinical trial, Urushima et al. investigated the effect of vitamin D supplements during winter on the incidence of seasonal influenza A in schoolchildren. In this study, influenza A occurred in 10.8% of children in the vitamin D group compared with 18.6% in the placebo group, suggesting that vitamin D supplementation may reduce the incidence of influenza A [158].

Although the precise immunomodulatory mechanisms of vitamin D are still being discovered, vitamin D supplementation may hold therapeutic promise in many diseases characterized by inflammation, including malignancies, cardiovascular diseases, autoimmune disorders and infections. Previous to the work described in this thesis, the impact of vitamin D on immune responses to the pneumococcus was unknown. However, in paper III, we speculate that vitamin D might be useful as an immunomodulatory drug, targeting the inflammatory response in pneumococcal disease.

1.2.4 Primary Immunodeficiencies

Primary immunodeficiencies (PIDs) are genetic disorders in which part of the immune system is missing or does not function properly. Most PIDs are diagnosed in young children, although milder forms may not be recognized until adulthood. Currently, more than 150 PIDs are recognized [159], and these can be divided into subgroups based on the component of the immune system that is affected. Patients with PID disorders are susceptible to infections that, if left untreated, may be fatal. The standard therapy for PIDs that include antibody deficiencies is intravenously administered immune globulin (IVIG). Many patients are also managed with antibiotic prophylaxis and some are given immunizations for encapsulated bacteria, like the pneumococcal conjugate vaccine. Nevertheless, many patients still experience frequent infections and more research is needed to elucidate the immunological abnormalities.

For the purpose of this thesis, two disorders; Hyper-IgE syndrome (HIES) and common variable immunodeficiency (CVID), will be described.

HIES

HIES is a PID characterized by recurrent staphylococcal skin abscesses and severe pulmonary infections. Dominant-negative mutations in *STAT3*, a transcription factor that mediates signaling of a multitude of cytokines and growth factors, have been identified as a major molecular cause of HIES [160]. Since *STAT3* plays a critical role in the signal transduction of many cytokines, it is difficult to determine which pathway is critical for the signs of HIES. Many reports have indicated that the production of Th17 cytokines, including IL-17, from HIES patients is much lower than those from

control individuals [160-162], suggesting that the impaired development of Th17 cells may account for the immunological abnormalities of HIES. However, these findings raise the question of why systemic Th17 cell defects lead to the unique susceptibility to particular infections, confined to the skin and lung, seen in HIES patients. More research into the molecular mechanisms underlying HIES will hopefully open up possibilities for exploring new approaches to treat these patients.

CVID

CVID is the most commonly encountered PID in clinical practice (for a review, see [163]). It represents a heterogeneous collection of disorders resulting mostly in antibody deficiency and recurrent infections. The low levels of antibodies (IgG, IgA and/or IgM) render patients susceptible to common bacterial and viral infections. In addition, patients respond poorly to vaccinations with protein and polysaccharide antigens such as the pneumococcal vaccine. Approximately 50% of patients with CVID also have T cell dysfunction. In recent years, significant progress has been made in elucidating genetic mechanisms that can result in a CVID phenotype; however, there still remains significant work to be done in improving our understanding of the disease.

1.3 BACTERIA HOST INTERACTIONS

S. pneumoniae is a fascinating bacterium from an immunological point of view. As a commensal of the human respiratory tract, it normally colonizes individuals without causing any symptoms. However, when it transmigrates into the lungs, enters the blood stream or crosses the blood-brain barrier, it transforms from a harmless colonizer to a serious pathogen able to cause life-threatening diseases. The host immune response to pneumococci must be balanced in order to induce sufficient clearance of bacteria while at the same time limit tissue damage. Nevertheless, the pneumococcus has a range of factors that enable it to evade immune defenses and cause disease.

1.3.1 Pneumococcal Components

A large number of pneumococcal factors related to host interactions have been described. These components are often called virulence factors. However, there are limitations to the concept of virulence, which assumes that bacteria are equipped with factors to harm the host. It is difficult to define virulence factors in the absence of host factors and the host response [164], and hence, the concept of virulence works for some bacterial pathogens but may be less suited for commensal bacteria with pathogenic potential, like the pneumococcus.

Polysaccharide Capsule

Pneumococci are encased by a capsular polysaccharide and the capsule is recognized as a *sine qua non* for invasive diseases. It is possible that host recognition of highly conserved motifs on the bacterial surface has pushed microorganisms to surround themselves with capsules, which can hide their underlying sensitive structures from host detection. The capsule protects pneumococci from phagocytosis by immune cells and is essential for survival in the blood. On the other hand, expression of the capsule can be downregulated during colonization of epithelial cells [165]. This may be beneficial for the bacterium since lower amounts of capsule enhances adherence and may promote colonization.

Cell Wall

The cell wall of pneumococci is built up by several layers of peptidoglycan and is rich in teichoic acids (TA) and lipoteichoic acid (LTA). Another important feature of the pneumococcal cell wall is choline, to which many pneumococcal proteins are anchored (**Figure 5**).

Peptidoglycan is constituted of glycan chains made of N-acetylglucosamine and N-acetylmuramic acid disaccharide subunits which are linked to peptide stems.

Peptidoglycan induces immune activation, and originally it was thought to signal via TLR2 [166, 167]. However, more recently, it has been shown that small quantities of LTA are present in commercial peptidoglycan preparations and that if the preparations are repurified to eliminate LTA, the TLR2-dependent activity of peptidoglycan is abolished [168].

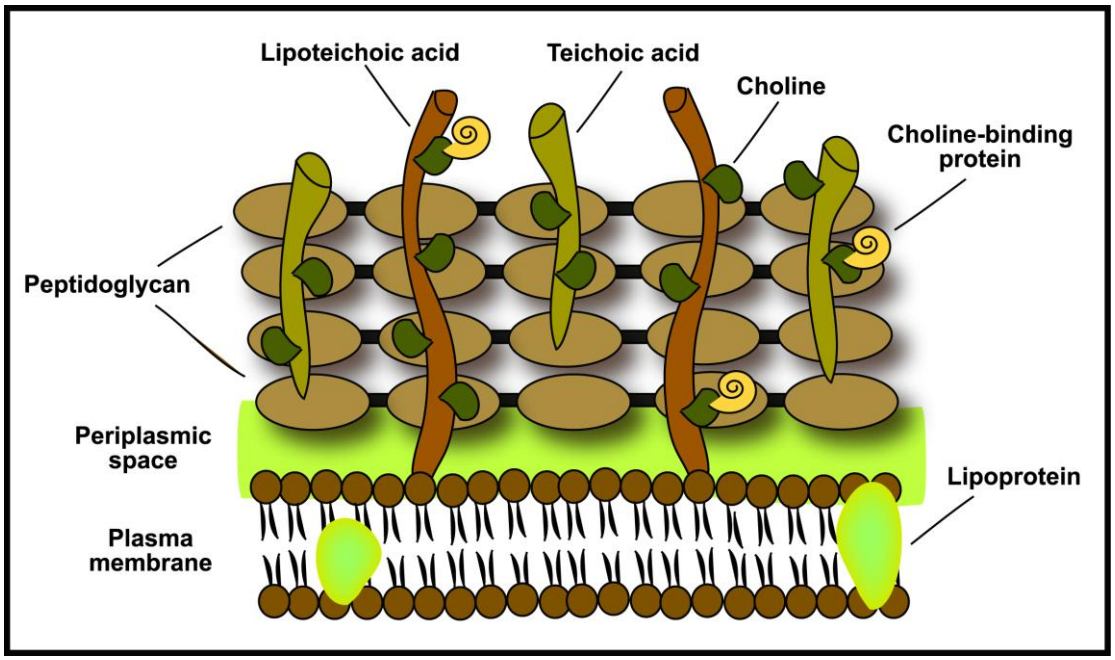

Figure 5. The pneumococcal cell wall. The pneumococcal cell wall consists of peptidoglycan, teichoic acid, lipoteichoic acid, choline and choline-binding proteins. © M. Olliver

Muropeptides are breakdown products of peptidoglycan which are released during bacterial growth and division. They are also released after phagocytosis as part of the host response, triggering intracellular signaling cascades and activation of the immune response.

Muramyl dipeptide (MDP) is a prominent muropeptide, known since the 1970s to be the minimal structure that displays adjuvant activity [169]. It is recognized by the cytoplasmic receptor NOD2 [110]. Some authors have found that MDP induces cytokine release from immune cells [170] whereas others have reported that it does not [171]. Possible explanations for the different results could be different origin and purity of the muropeptides, contamination by LPS, or the unphysiologically high concentrations of MDP used [172]. Notably, Iyer and Coggeshall recently showed that intact polymeric peptidoglycan is a better stimulator of human innate immune cells than peptidoglycan monomers such as MDP, and that the polymeric nature of peptidoglycan is required for efficient phagocytosis and lysosomal degradation [173].

Surface Proteins

Pneumococci possess more than 500 surface proteins. Three main groups have been identified: lipoproteins, LPXTG-proteins, and choline-binding proteins (CBPs). These

proteins enable the bacterium to colonize and adhere to the nasopharyngeal and respiratory tract epithelium. They are also involved in bacterial fitness, cell-cell contact, elimination of competitors and host interaction. During the various stages of the infection, when pneumococci have to adapt to different niches, expression of these proteins occurs in a highly regulated manner [174].

Pneumolysin

Pneumolysin, a 53 kDa protein that is present in virtually all clinical isolates of *S. pneumoniae*, is one of the most widely studied pneumococcal factors. At high doses, the toxin binds to and creates large pores in cholesterol-containing host cell membranes [175], causing host cell lysis and death [86, 176]. At low doses, recombinant pneumolysin has been shown to activate the complement system [177], inhibit chemotaxis of neutrophils [178], and induce cytokine production from human monocytes and neutrophils [179, 180]. Recombinant pneumolysin was also shown to cause damage to human ciliated epithelium *in vitro* [181].

It has previously been believed that pneumolysin is an intracellular protein that is only released when cells undergo autolysis [182], but a more recent report showed that pneumolysin is primarily localized to the cell wall compartment during growth in the absence of detectable cell lysis [183].

The role of pneumolysin in the pathogenesis of infection has been widely studied in animal models. Mice infected with a pneumolysin-deficient strain showed reduced lethality and diminished inflammatory responses in the lungs when compared to wild type [182, 184-186]. In otitis media and meningitis models, however, others have reported that pneumolysin does not have an effect on inflammation [187, 188]. Furthermore, clinical isolates expressing non-hemolytic forms of pneumolysin have been detected in IPD patients [189, 190], demonstrating that the hemolytic activity of the toxin is not essential for invasive disease. In addition, there has also been a report of a clinical isolate with no pneumolysin expression [191]. Hence, although many animal studies have established pneumolysin as an important virulence factor, the picture is not clear**.**

While murine models have been invaluable for studying pneumococcal pathogenesis, care should always be taken in extrapolating the results from murine cells to the human response. In addition, studying the effects of isolated recombinant pneumolysin may not give the same results as looking at the more complex interactions between whole bacteria and host cells. Furthermore, deletion of a single virulence factor to determine the contribution of this factor to the overall virulence phenotype can be difficult. Pneumococci possess numerous attributes that contribute to virulence and tools to study the combination of virulence factors simultaneously are needed.

1.3.2 Naturally Acquired Protection against *S. pneumoniae*

Both the prevalence of pneumococcal colonization and the incidence of pneumococcal disease in unvaccinated children decline over the course of childhood [6, 192, 193]. The reason for the changing susceptibility to pneumococcal carriage and disease after the first years of life is not fully understood. Potential mechanisms that underlie naturally acquired protection against pneumococci will be described here.

Age-related maturation of the immune system
Age-related changes in susceptibility to infections may be due to maturation of the immune system. Bogaert et al. showed that similar to what is observed in humans, the clearance of pneumococcal colonization in infant mice is significantly impaired compared to that in adult mice [50]. In the human system, differences in TLR-mediated cytokine responses in cells from infants compared to adults have been reported [194, 195]. These studies indicate that when the immune response matures with age, it becomes increasingly capable of mounting innate and adaptive responses to pneumococci.

Humoral immunity by antibody directed against the capsule
It has generally been thought that the immune response to pneumococci in naturally exposed, unvaccinated children depends primarily on antibodies directed against the polysaccharide capsule. The reason for this is that immunization with the polysaccharide and conjugate vaccines generate effective serotype-specific antibody responses [196, 197]. In addition, patients with antibody deficiencies are at increased risk of pneumococcal disease [198]. Immunity by serotype-specific opsonic antibodies is thought to mediate protection by enhancing the bactericidal effect of host phagocytes.

Relatively few data exist on immune responses to the pneumococcus after natural exposure. Some of these studies have presented evidence for serotype-specific acquired immunity. Weinberger et al. analyzed longitudinal carriage data from Israeli toddlers attending day care, and observed a serotype-specific acquired immunity to pneumococcal carriage in some of the serotypes investigated [199]. There was a lower risk of colonization with serotypes 6A, 14 and 23F after previous exposure to the homologous type. They found no evidence of specific protection for serotypes 6B, 15 and 19F. However, this type of observational study has some limitations. For example, when subjects were sampled, the serotype of only one colony was determined and since multiple serotypes can potentially colonize the same individual, the dominant type would most likely be the one that is detected. Another study, in adults, showed that levels of anticapsular antibodies increase significantly after carriage of some, though not all, serotypes [6].

However, lately the assumption that anticapsular antibodies are the primary mechanism of naturally acquired immunity to pneumococci has been questioned. The timing of

protection appears before anticapsular antibody responses can be detected in most children, and there is a simultaneous decline in carriage and disease for many serotypes, which would be unlikely if separate antibody responses were required for protection against each serotype [192]. In addition, in an experimental challenge study in adults there was no evidence of an association between prechallenge anticapsular antibody concentration and protection [200]. Furthermore, Granat et al. found that previous pneumococcal carriage was associated with a serotype-independent protection from subsequent acquisition in Bangladeshi children [201], consisted with the hypothesis that serotype-independent immunity is stimulated in young children by previous pneumococcal carriage. Finally, McCool and Weiser reported that clearance of pneumococcal carriage in mice could occur independently of antibody [202], suggesting that other components of the immune response may play a role.

Antibody-independent T cell-dependent cellular immunity

In 2004, Kadiouglu et al. showed that mice with a deletion of the MHC-II gene, which are effectively CD4-T-cell negative, are significantly more susceptible to pneumococcal bronchopneumonia and septicemia than their isogenic wild-type parents [203]. Since then, a number of papers have reported observations relevant to the role of $CD4^+$ T cells in the host immune response to pneumococcal infection [103, 136, 204, 205].

The Th17 cell response to pneumococci is increasingly recognized as an important mediator of immunity and several mouse studies have shown the importance of these responses in inducing neutrophil and monocyte/macrophage mediated clearance of colonizing bacteria [136, 137]. IL-17-mediated immunity to pneumococcal colonization was also observed in mice immunized intranasally with the pneumococcal cell wall polysaccharide [206]. However, in a recent study by Cohen et al., protection against invasive pneumococcal infection in mice was due to serum antibody responses (that promoted clearance of pneumococci from the blood) and not a CD4-dependent IL-17 response [138], suggesting that the protective Th17-mediated effects of nasopharyngeal colonization may depend on the site of subsequent challenge.

There are contradictory reports on the role of the Th1 cytokine IFN-γ in protection against pneumococcal infection in murine models [207, 208] . Nevertheless, Kemp et al. found that IFN-γ producing T cells disappear from the circulation in patients with pneumococcal pneumonia, suggesting that these cells are engaged in the immune response [209].

Few studies have explored human $CD4^+$ T cell responses to pneumococci. Mureithi et al. showed that following challenge with pneumococcal antigens, human PBMCs produce IL-17 and IFN-γ *in vitro* [139]. Recently, Pido-Lopez et al. demonstrated an age-related sequestration of Th1 and Th17 $CD4^+$ T cells reactive to pneumococcal protein antigens within mucosal lymphoid tissue [210]. Their experiments also revealed the presence of antigen-specific regulatory T cells, suggesting that the

balance between mucosal effector and regulatory Th cell immunity is critical to pneumococcal commensalism.

A better understanding of the mechanisms by which pneumococci trigger human Th cell responses may facilitate the design of new pneumococcal vaccines based on cell-mediated immunity.

2 AIMS

The overall aim of this thesis was to explore the complex interactions between *S. pneumoniae* and its human host. Focus has been on human dendritic cells (DCs) and the innate and adaptive immune responses they induce after infection with live pneumococci. An improved understanding of host defense mechanisms against pneumococci might lead the way for the development of novel therapeutic strategies to regulate immune responses and inflammation.

The specific aims of the papers were:

- To study the response of human DCs to a live pneumococcal infection and investigate the effect of the pneumococcal cytotoxin pneumolysin on DC viability, maturation and production of inflammatory cytokines. Furthermore, to compare the responses of human and murine DCs. (**Paper I**)

- To investigate the role of monocytes in promoting Th responses to live and heat-killed pneumococci. Also, to identify pneumococcal components responsible for triggering production of Th1 and Th17 effector cytokines. (**Paper II**)

- To further explore the interactions of pneumococci and DCs, as well as mechanisms underlying pneumococcus-induced Th responses. In addition, the objective was to examine the immunological effects of vitamin D on immune functions in response to pneumococci. (**Paper III**)

- To determine whether patients with PIDs exhibit impaired production of AMPs and whether this could be linked to an aberrant Th17 response. (**Paper IV**)

3 METHODOLOGICAL AND ETHICAL CONSIDERATIONS

This chapter contains information about the bacterial strains and cells used in **papers I-IV**. For more details about the *in vitro* infection assays, see the Materials and Methods sections of the respective papers.

3.1 BACTERIA

Throughout this work, the serotype 4 strain T4 (TIGR4, ATCC BAA-334) and its non-encapsulated mutant T4R were used. The strain T4 was originally isolated from the blood of a Norwegian patient and its genome was sequenced in 2001 [211]. T4 is commonly used in laboratories around the world and shows a high level of virulence in mice [106].

Virtually all clinical isolates of *S. pneumoniae* contain an external capsule. However, in our initial *in vitro* infection assays with human DCs, using serotype 4 pneumococci, we found that only the unencapsulated strain T4R was readily taken up by the cells. Significant uptake of the encapsulated parent strain T4 was only observed under opsonized conditions, high infection doses or long infection times. For this reason we used T4R in many of the experiments. The rationale for this was that we anticipated that the results observed with unencapsulated T4R in our studies would reflect the physiological (*in vivo*) conditions with opsonised uptake of encapsulated pneumococci.

In **paper I**, **II and III**, T4 and T4R mutants lacking pneumolysin were used. These mutants were created by insertion-deletion mutagenesis, and the targeted gene was replaced by an erythromycin-resistance cassette.

In **paper I**, a number of clinical isolates of serotype 1 were used. These belong to the sequence type (ST) 306, producing a non-hemolytic form pneumolysin, as well as ST228, ST217 and ST227, producing hemolytic pneumolysin. All clinical isolates were obtained from Birgitta Henriques-Normark at the Swedish Institute for Infectious Disease Control, Department of Bacteriology. In **paper II**, clinical isolates of serotype 9 and 14 were also used, as well as the serotype 2 strain D39 (obtained from Peter Hermans, Rotterdam, The Netherlands).

Pneumococci were grown over night on blood agar plates at 37˚C in 5% CO_2. Colonies were grown in C+Y medium (Karolinska University Laboratories) to mid-log phase, then pelleted by centrifugation and diluted with cell culture medium prior to infection of cells. In some experiments, bacteria were heat-killed by incubation of cultures for 20 minutes at 90˚C. Killing was confirmed by plating bacteria on blood agar plates.

3.2 CELLS

The majority of experiments included in the papers of this thesis were performed with primary immune cells, isolated from the blood of human donors.

For **paper IV**, ethical approval for blood collection from patients and healthy controls was obtained from the research ethical committee at Karolinska Institutet, and written informed consent was obtained from all patients prior to inclusion and sample collection. Approval for the generation of murine DCs, for **paper I**, was obtained from the ethical committee on animal research of Stockholm, section north.

Monocyte-derived DCs
In **paper I** and **III**, monocyte-derived DCs were used. Buffy coats were obtained from healthy volunteers, provided by Karolinska University Hospital. PBMCs were isolated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare) and monocytes were obtained either by negative selection with RosetteSep human monocyte enrichment cocktail (StemCell Technologies) (paper I), or by incubation of PBMCs to a large plastic culture flask where monocytes were allowed to adhere for two hours (paper III). Monocytes were then cultured for 6 days in cell culture medium in the presence of GM-CSF and IL-4. The DC phenotype was assessed for surface marker expression ($CD14^-$, $CD11c^+$ and $CD1a^+$) with fluorescently labeled antibodies.

Murine bone marrow-derived DCs
In **paper I**, we used murine bone marrow-derived DCs, to compare some of the responses to human DCs. Primary cells were obtained from the bone marrow of 6-10 week old C57BL/6 and BALB/c mice and the cells were differentiated for 6 days in the presence of recombinant murine GM-CSF. The phenotype of the cells was assessed by examining the expression of CD11c.

Murine cells are commonly used in pneumococcal infection studies. However, as mice are not natural hosts for pneumococci, receptor expression and cellular composition in these cells may differ from human cells, and care must be taken to directly extrapolate the results from murine cells to the human response.

Monocytes
Monocytes were used in **paper II**. Buffy coats were obtained from healthy volunteers, provided by Karolinska University Hospital. PBMCs were isolated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare) and monocytes were purified by negative selection with RosetteSep human monocyte enrichment cocktail (StemCell Technologies). Monocyte purity was assessed by examining CD14 expression.

$CD4^+$ T cells
In **paper II** and **III**, $CD4^+$ T cells were used in co-cultures with monocytes or DCs. Unfractionated $CD4^+$ T cells were purified from buffy coats by negative selection with

RosetteSep human $CD4^+$ T cell enrichment cocktail (StemCell Technologies), and memory and naïve $CD4^+$ T cells were prepared with EasySep human memory $CD4^+$ T cell enrichment kit and EasySep human naïve $CD4^+$ T cell enrichment kit (StemCell Technologies), respectively. The purity of the cells was assessed by examining expression of CD3, CD4 (unfractionated $CD4^+$ T cells) as well as CD45RO (memory $CD4^+$ T cells) and CD45RA (naïve $CD4^+$ T cells) with fluorescently labeled antibodies.

PBMCs

In **paper IV**, PBMCs were isolated from whole blood of patients and healthy controls by density gradient centrifugation (Lymphoprep). The PBMCs were cryopreserved and on the day of the experiment, cells were quickly thawed in a 37˚C water bath, washed twice and resuspended in cell culture medium.

Detroit 562 (D562) nasopharyngeal epithelial cells

In **paper III**, a human pharynx carcinoma cell line, called D562, was used for analysis of IL-8 mRNA expression. The advantage of using a cell line is that it is possible to perform several experiments with cells of the same origin. However, immortalized cells may express different receptors from primary cells which creates concerns about the biological relevance.

4 RESULTS AND DISCUSSION

4.1 PAPER I

"*Streptococcus pneumoniae* Evades Human Dendritic Cell Surveillance by Pneumolysin Expression"

In paper I, published in 2009, we investigated the dynamics of human DC interaction with live pneumococci, with a focus on the pneumococcal cytotoxin pneumolysin. Using *in vitro* infection assays, we examined uptake and intracellular processing of pneumococcal strains by the DCs with gentamicin-protection assays and transmission electron microscopy (TEM). We also examined DC viability and functions such as expression of maturation markers and cytokines, using flow cytometry and ELISA analyses. In addition, we compared some of the human DC responses to those of murine DCs.

Our assays revealed that unencapsulated pneumococci are readily taken up by DCs. Significant uptake of the encapsulated parent strain was only observed under opsonized conditions, high infection doses or long infection times. We found that uptake of a pneumolysin-deficient mutant was lower than for the pneumolysin-producing parent strain, suggesting that bacteria-bound pneumolysin interacts directly with host cell receptors. However, the specific receptors and mechanisms involved need to be further examined.

In addition, we showed that production of pneumolysin affects intracellular processing of pneumococci by the DCs. At 4 hours p.i., the majority of pneumolysin-producing bacteria appeared in tight vacuoles, whereas pneumolysin-deficient bacteria were found in both spacious and tight vacuoles (**Figure 6**). Nevertheless, at 8 hours p.i., both pneumococcal strains were localized in tight vacuoles. These results indicate that pneumolysin may be involved in the transition of bacteria from spacious to tight vacuole. It appears that bacteria inside tight vacuoles in fact reside free in the cytoplasm; however, further characterization of these compartments is required to examine the degree of contact between bacteria and the cytosol.

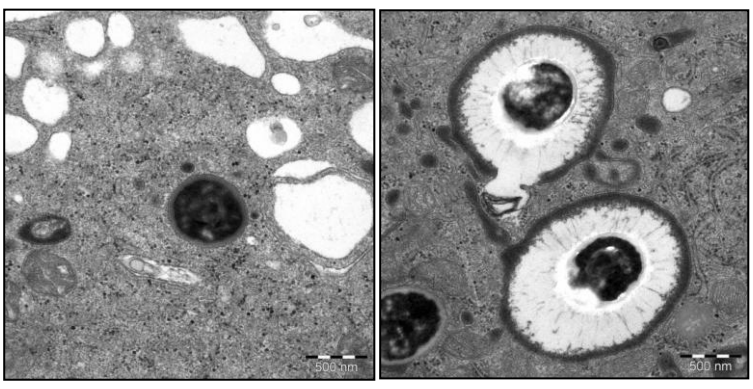

Figure 6. Pneumococci reside in two types of vacuoles following uptake. Left: Pneumolysin-producing pneumococcus in a tight vacuole in close contact with the cytosol. Right: Pneumolysin-deficient pneumococci in spacious and tight vacuoles. TEM pictures by Kjell Hultenby.

Infection with pneumolysin-producing pneumococci activated multiple caspases and induced significant apoptosis of the infected DCs. The apoptotic effect of pneumolysin

was more pronounced in the unencapsulated background, suggesting that it is dependent on an intracellular location. Unexpectedly, we found that pneumolysin diminished activation of the DCs. Infection with pneumolysin-producing pneumococci resulted in significantly lower expression of the surface activation markers CD80 and CD86 in live DCs compared to the pneumolysin-deficient mutant.

Several investigators have reported that exposure of human immune cells to recombinant pneumolysin induces inflammatory cytokines [179, 180]. However, in our experiments with human DCs, using intact bacteria, we found that pneumolysin-deficient pneumococci induce significantly higher levels of the inflammatory cytokines IL-12p70, IL-8 and IL-1β compared to wild type. Interestingly, the 30-fold increased amount of IL-12p70 induced by the pneumolysin-deficient strain could not be solely attributed to higher cell viability, which was only 3-fold. Instead, we speculate that an increased early signaling to vacuolar receptors leads to the enhanced inflammatory response, since pneumolysin-deficient bacteria were located within spacious vacuoles for an extended period after infection.

In addition to the serotype 4 pneumolysin knock-out mutant, we also performed experiments with clinical isolates of serotype 1, expressing non-hemolytic forms of pneumolysin [190]. Pathogenic serotype 1 isolates producing non-hemolytic pneumolysin are frequently isolated from sterile sources [189], demonstrating that the hemolytic activity of the toxin is not essential for invasive disease. Similar to the serotype 4 pneumolysin-deficient mutant, these strains were more proinflammatory and caused less apoptosis than clonally related strains expressing hemolytic pneumolysin.

Most studies examining the impact of pneumolysin have been performed with murine cells. Our results are in contrast to the previously reported proinflammatory nature of pneumolysin in murine macrophages [212]. Therefore, we wanted to compare the cytokine response to pneumococci between human and murine DCs following infection with pneumolysin +/- strains. Interestingly, the production of IL-12p70 followed the same pattern in human and murine DCs, whereas in murine cells IL-1β was only induced following infection with pneumolysin-producing bacteria. Thus, we could repeat what has previously been reported, namely that pneumolysin can stimulate a proinflammatory response. Importantly, these results highlight the need to study human responses to this human-specific pathogen.

To conclude, this paper shows that the pneumococcus possesses the capacity to evade human DC-mediated immune responses. Firstly, expression of the capsule inhibits internalization, thus reducing intracellular sensing of pneumococcal ligands. Secondly, pneumolysin inhibits DC-mediated immunosurveillance such as cytokine production and co-stimulation of T cells. Thirdly, the observed induction of apoptosis by pneumolysin-producing strains may compromise the development of DC-mediated adaptive immune responses against pneumococci. Taken together, modulation of DC-induced immunity may be a strategy by which the pneumococcus promotes colonization and disease.

4.2 PAPER II

"Human Monocytes Promote Th1 and Th17 Responses to *Streptococcus pneumoniae*"

Natural immunity to pneumococcal infections has been assumed to depend on anticapsular antibodies. However, recent findings from murine models suggest that alternative mechanisms, which are dependent on Th cells, are also involved [202, 204, 205]. Since the immunological events in which Th cells contribute to immunity have mainly been studied in mice, we wanted to examine how these responses are generated in the human system. Consequently, in paper II, published in 2011, we investigated bacterial and host factors involved in the induction of Th1 and Th17 responses, using a co-culture model of human monocytes and $CD4^+$ T cells.

We could show that pneumococcus-infected monocytes promote effector cytokine production by memory Th cells, leading to a mixed Th1/Th17 (IFN-γ/IL-17) response. Interestingly, we found that live and heat-killed pneumococci induced distinct cytokine responses and that different mechanisms were involved in the promotion of Th1 and Th17 cytokines. Accordingly, live bacteria triggered a Th1-biased response that was a 100-fold higher than that to heat-killed bacteria. IFN-γ production was dependent on monocyte production of IL-12 and was enhanced in the absence of the pneumococcal capsule and the presence of human serum. Furthermore, we found that live bacteria were phagocytosed 20-fold more than heat-killed bacteria. Taken together, these results suggest that the Th1 dominant response is induced by the presence of internalized viable pneumococci.

Heat-killed bacteria triggered Th17 cytokine production through TLR2 signaling; however, our results showed that this receptor is not involved in the Th response to live bacteria. It has previously been shown that internalized pneumococci trigger activation of NOD2 [111, 112], though it remains to be elucidated if NOD2 is involved in the promotion of Th cytokines in response to live pneumococci in our system.

Live pneumococci triggered a dose-dependent increase in IFN-γ, but not IL-17, and we asked ourselves if this was because live bacteria could induce Th1-skewing conditions that prevent the induction of a Th17 response. To test this hypothesis, we added exogenous recombinant IL-12p70 to cocultures infected with heat-killed bacteria, known to trigger substantial levels of IL-17. This led to a significant decrease in IL-17, suggesting that IL-12 has an important role in establishing the Th1/Th17 balance. A negative relationship between Th1 and Th17 cytokines has previously been reported [213, 214].

In an effort to elucidate which pneumococcal factors were responsible for the Th cytokine response, we examined the influence of various pneumococcal components. In contrast to a previous study by McNeela et al. [215], we found no difference in the induction of IFN-γ and IL-17 between wild type pneumococci and a pneumolysin-

deficient mutant. Capsular type and the capsule itself did not appear to be important. Rather, our results suggested that pneumococcal peptidoglycan is a potent trigger of both Th1 and Th17 cytokines.

To summarize, this work provides evidence that pneumococcal peptidoglycan triggers the production of Th1 and Th17 cytokines from human memory cells, and that the balance between the two immune effector arms depends on bacterial viability. An increased understanding of human Th responses is essential for the development of pneumococcal vaccines designed to elicit cell-mediated immunity. A deeper understanding of the pathways that regulate Th cytokines may also be used to develop immunomodulatory therapies to suppress excessive mucosal inflammation in pneumococcal infections.

4.3 PAPER III

"Human dendritic cells, activated by pneumococcal peptidoglycan, promote innate and adaptive immune responses which can be modulated by vitamin D"

DCs play a central role in regulating innate and adaptive immune responses against commensal and pathogenic bacteria. However, little is known about what drives DC-mediated immune responses to pneumococci in humans. Paper III, a manuscript, aims at examining the ability of human DCs to induce innate and adaptive immune responses to the pneumococcus. In addition, we investigated the immunomodulatory effects of vitamin D on DC-mediated immune functions.

We showed that uptake of live pneumococci and pneumococcus-derived purified peptidoglycan induce DC maturation and cytokine production. Supernatants from stimulated DCs induced an inflammatory IL-8 response in nasopharyngeal epithelial cells, measured as an increase in mRNA levels. DCs co-cultured with $CD4^+$ T cells promoted Th1 and Th17 memory cytokines (IFN-γ and IL-17), in a cytokine and contact-dependent manner. These results suggest a role for Th1 and Th17 cells in establishing anti-pneumococcal immunity.

Vitamin D is a well-known immunomodulator [149, 151]; however, nothing is known about the impact of vitamin D on pneumococcal immune responses. In our experiments, we found that addition of vitamin D enhanced maturation of DCs and skewed the Th response from an inflammatory Th1/Th17 phenotype towards a regulatory T cell phenotype. These data indicate that vitamin D regulates the immune balance in response to pneumococci by mitigating inflammation caused by the adaptive immune response. Although local inflammatory responses may be beneficial by preventing the pneumococcus from invading mucosal tissues and cause disease, excessive inflammation may result in tissue damage. Our findings suggest that vitamin D might be useful as an immunomodulatory drug, targeting pneumococcus-induced inflammation.

To conclude, our results demonstrate that memory $CD4^+$ T cells responding to *S. pneumoniae* stimulation can be detected in the circulation of healthy donors, and that pneumococcal peptidoglycan is a potent trigger of DC-mediated immune responses. An improved understanding of the interactions between human host cells and pneumococci may lead to development of novel therapeutic strategies, including regulation of immune responses and inflammation.

4.4 PAPER IV

"Impaired Release of Antimicrobial Peptides into Nasal Fluid of Hyper-IgE and CVID Patients"

Patients with PIDs often suffer from frequent respiratory tract infections, despite standard treatment with IVIG and antibiotics. In paper IV, published in 2011, we sought to investigate if additional immunological abnormalities are present among patients with various PID diagnoses. Two key components of the innate and adaptive immune system were examined, namely induction of AMPs (LL-37 and HNP1-3) and Th17 cells (IL-17).

We collected nasal fluid, nasopharyngeal swabs and PBMCs from patients as well as healthy controls. AMP levels were measured in nasal fluid and nasal swabs were cultured for bacteria. PBMCs were stimulated with antigen and the supernatant was assessed for IL-17 release.

We could show that healthy controls and most patients colonized with a primary pathogen, such as *S. pneumoniae*, exhibited increased levels of AMPs in nasal fluid compared to individuals with a negative bacterial culture. However, there was no increase in neither LL-37 nor HNP1-3 in CVID and HIES patients, despite growth of pathogenic bacteria.

Th17 cells are instrumental in mucosal immunity by orchestrating the production of AMPs in epithelial cells as well as by recruiting neutrophils to mucosal tissues [216]. To investigate if lack of AMP expression in nasal fluid in CVID and HIES patients could be correlated with an impaired release of IL-17 from circulating immune cells, we measured IL-17 in antigen-stimulated PBMCs. Milner et al. previously showed that naive T cells from patients suffering from HIES cannot differentiate into Th17 cells [162]. In accordance with these results, we found that PBMCs from HIES patients did not produce IL-17 in response to antigenic stimuli. Interestingly, CVID patients also exhibited an impaired production of IL-17, which has not previously been reported. These results warrant further investigations to elucidate the mechanisms involved.

Taken together, this investigation presents evidence that suggest that CVID and HIES patients have a dysregulated AMP response to pathogenic bacteria in the upper respiratory tract, which could be linked to an aberrant Th17 cell response. Our data also suggest that patients with certain PIDs may benefit from AMP-inducing agents such as vitamin D.

5 CONCLUDING REMARKS

DCs are the most potent APCs of the immune system. They play a central role in regulating the nature of innate and adaptive immune responses against bacteria, both in health and pathology. Despite this, little is known about what drives DC responses to the pneumococcus in humans. In the work included in this thesis, we report that human DCs orchestrate a repertoire of immune responses upon sensing intracellular pneumococci and pneumococcus-derived peptidoglycan. Our findings provide new insights into mechanisms used by the pneumococcus to evade host-sensing systems, and call for further studies on the potential role of anti-inflammatory therapeutic strategies for pneumococcal diseases.

Although the pneumococcus has been researched by microbiologists for over 80 years, we still understand relatively little about this organism as a commensal and pathogen. Controlling pneumococcal disease is a massive challenge that will require a multifaceted approach. Clinical evaluation of novel vaccine approaches (based on pneumococcal proteins or killed whole cells) will be of great interest, as well as development immunomodulatory therapies targeting the proinflammatory events thought to underlie the pathogenesis of invasive pneumococcal disease.

Naturally acquired protection against pneumococci appears to be responsible for the lower rates of carriage and disease in older children and young adults. Hence, the key to rational strategies for control of pneumococcal disease is a better understanding of how natural immunity to the pneumococcus is developed.

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